Formation of Supported Phospholipid Bilayers on Molecular Surfaces: Role of Surface Charge Density and Electrostatic Interaction

TaeWoon Cha,* Athena Guo,† and X.-Y. Zhu*

*Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455; and †MicroSurfaces, Inc., Minneapolis, Minnesota 55421

ABSTRACT Electrostatic interaction is known to play important roles in the adsorption of charged lipids on oppositely charged surfaces. Here we show that, even for charge neutral (zwitterionic) lipids, electrostatic interaction is critical in controlling the adsorption and fusion of lipid vesicles to form supported phospholipid bilayers (SPBs) on surfaces. We use terminally functionalized alkanethiol self-assembled monolayers (SAMs) to systematically control the surface charge density. Charge neutral egg phophatidylcholine (eggPC) vesicles readily fuse into SPBs on either a positively charged 11-aminino-1-undecanethiol SAM or a negatively charged 10-carboxy-1-decanethiol SAM when the density of surface charge groups is ≥80%. These processes depend critically on the buffer environment: fusion of adsorbed vesicles to form SPBs on each charged molecular surface does not occur when the molecular ion of the buffer used is of the opposite charge type. We attribute this to the high entropic repulsion (electric double layer repulsion) due to the large size of molecular counterions. On the other hand, such a critical dependence on buffer type is not observed when charged lipids are used. This study suggests the general importance of controlling electrostatic interaction in the formation of stable SPBs.

INTRODUCTION

Supported phospholipid bilayers (SPBs) have received increasing attention due to their applications in biosensors and their importance as models for biological membranes (1–4). A number of studies have characterized the spontaneous fusion of adsorbed vesicles to form SPBs on oxides and polymeric supports by quartz crystal microbalance, atomic force microscopy, and fluorescence microscopy (5–7). To mimic natural membranes, it is necessary to reconstitute membrane proteins into an SPB in a mobile form. A key factor is the two-dimensional mobility of lipid molecules within the SPB. It has been shown that SPBs formed on oxide (SiO_2 and TiO_2) surfaces possess lateral fluidity, but they are unstable when withdrawn from the air/water interface (5,8). In contrast, the use of polymer-cushioned substrates increases the stability of SPBs while reducing long-range lateral mobility of lipid molecules (9,10). Recently, Cremer and co-workers reported a protein-covered lipid bilayer system which is both stable upon exposure to the air and completely fluidic in lateral diffusion (11). These studies point to the importance of surface-vesicle interactions, including van der Waals, electrostatic, hydration, and steric forces, in SPB formation. A number of studies have addressed mechanistic aspects of vesicle fusion and SPB formation by varying pH, ionic strength, charge contents in lipids, and the concentration of bivalent metal ions (Ca^{2+} or Mg^{2+}) in solutions (6,12–14). Electrostatic interactions involving charged lipids (15), including the interaction between charged lipids and oppositely charged surfaces (6), are well known. However, little is known about the possible role of electrostatic forces for charge neutral lipids.

In this report, we aim to establish the role of electrostatic interaction in the adsorption of zwitterionic charge neutral lipid vesicles and their fusion to form SPBs on surfaces. We focus on the effect of surface charge density on vesicle fusion and SPB formation. We use ω-functional alkanethiol self-assembled monolayers (SAMs) on gold as substrates for SPB formation. This system is chosen because it allows us to easily control surface charge density using mixed SAMs from different ω-functional alkanethiol molecules, namely 11-hydroxy-1-undecanethiol (HUD) mixed with 11-amino-1-undecanethiol (AUT) or 10-carboxy-1-decanethiol (CDT), as shown in Fig. 1 (16,17). We study the adsorption and fusion of small unilamellar vesicles (SUVs) on the SAM-covered surfaces by the fluorescence recovery after photobleaching (FRAP) technique. We focus on the dependences of vesicle fusion and lipid mobility as a function of surface charge density, controlled by the composition of mixed SAMs. Single component thiol SAMs on gold have been employed in past studies on vesicle adsorption and fusion (18,19).

MATERIALS AND METHODS

ω-Functionalized alkanethiol monolayer on gold

AUT (purchased from Dojindo Chemicals, Gaithersburg, MD), HUD, and CDT (both purchased from Aldrich, St. Louis, MO) were used as received. The gold substrates were prepared by thermal evaporation of 100 nm of Au onto polished Si(100) with a 10 nm Cr adhesion layer in a vacuum evaporator (10^{-6} torr). A deposition rate of 1 Å/s was used to obtain smooth Au surfaces. The freshly prepared Au/Si wafer was cut into 1.0 cm^2 pieces and immersed overnight in 0.2 mM (total thiol concentration) solutions of HUD with CDT, or AUT in ethanol. In the case of CDT solutions, 5% acetic acid was also added to the solution to avoid double layer formation (20). The

Submitted February 15, 2005, and accepted for publication November 3, 2005.

Address reprint requests to X.-Y. Zhu, E-mail: zhu@chem.umn.edu; or Athena Guo, E-mail: athena@memsurface.com.

0006-3495/06/02/1270/05 $2.00

doi: 10.1529/biophysj.105.061432

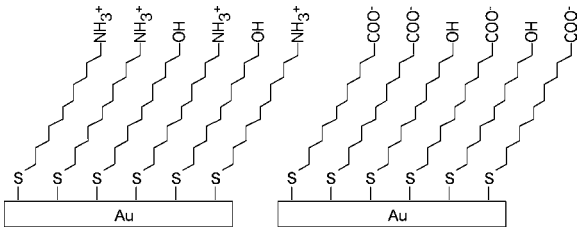

FIGURE 1 The use of mixed SAMs of ω-functional alkanethiols to generate positively charged (*left*) or negatively charged (*right*) surfaces.

composition of each SAM was confirmed by x-ray photoelectron spectroscopy. At pH = 7.5 used in all experiments, both SAM surfaces are in the charged form, i.e., $-NH_3^+$ and $-COO^-$.

Extruded lipid vesicles

EggPC (egg phophatidylcholine), DOTAP (1,2-dioleoyl-3-trimethyl ammonium propane), and Avanti Mini-extruder were purchased from Avanti Polar Lipids (Alabaster, AL). Texas Red tagged dihexadecanoyl-phosphatidylethanolamine (TR-DHPE) was purchased from Molecular Probes (Eugene, OR). All lipids were stored at −20°C until use. Preparation of SUVs was carried out via the extrusion method (Avanti Polar Lipids product catalog). Briefly, lipids from stock solution in chloroform were mixed with TR-DHPE and evaporated under argon flow at least for 3 h. The lipid mixtures were reconstituted in a buffer of interest (pH 7.5, 150 mM). The concentration was set at 1 mM (1 mol% of TR-DHPE). Suspension of the lipid mixtures was obtained by sonication and homogenized by prefiltering with 0.45 μm pores. The lipid suspension was forced through the polycarbonate filter with 30 nm pores more than 11 times. We determined the sizes of the vesicles to be in the range of 60–80 nm by dynamic light scattering using a homemade photometer equipped with a Brookhaven (Holtsville, NY) BI-DS photomultiplier and a Lexel Ar+ laser (Fremont, CA) operating at 488 nm. This SUV solution was stored at 4°C until use. The Tris (50 mM Tris (hydroxymethyl) aminomethane in 100 mM NaCl) and PBS buffers (150 mM, composed of NaCl, KCl, Na_2HPO_4, KH_2PO_4) used had the same ionic strength and pH (7.5). Purified water (18 MΩ·cm; Millipore, Billerica, MA) was used in all experiments.

Vesicle adsorption and FRAP measurements

A sufficient amount of the SUV solution was placed on each functionalized surface of interest and incubated for at least 2 h at room temperature. Excess vesicles were removed from the surface by flushing ~10 s with the same buffer used for the vesicle solution. A coverslip was placed on top of the surface to protect it from drying. The coverslips were precleaned with piranha solution (H_2SO_4/H2O2 = 3:1; caution: it is a strong oxidant and reacts violently with organic substances) and extensively washed with water before use. A confocal fluorescence microscope (LaserSharp MRC1024, BioRAD, Cambridge, MA) equipped with a krypton/argon laser was used for FRAP measurements. The excitation laser wavelength was 568 nm, and fluorescence intensity from the TR dye at 615 nm was detected/imaged. To start the FRAP experiment, we used full laser power and zoomed in (10×) to an area of 16.2 × 16.2 μm^2 to bleach the dyes in the illuminated area. We then zoomed out to an area of 162.1 × 162.1 μm^2 and obtained images after a certain time delay; we used low laser power in this step to ensure negligible bleaching during imaging. The time between bleaching and obtaining the first image was ~10 s. Each image was integrated for ~2 s.

RESULTS AND DISCUSSION

We use FRAP to characterize vesicle adsorption and SPB formation on each SAM-covered surface, as illustrated in Fig. 1. We use either positively or negatively charged surfaces from mixed SAMs of HUD mixed with AUT or CDT. If the SUVs adsorb intact as individual vesicles, there is no mobility after photobleaching. On the other hand, if adsorbed SUVs rupture to form a supported phospholipids bilayer, the rate of fluorescence recovery measures the mobility of lipids within the SPB. This study leads to the finding of two critical factors in the formation of a stable SPB from SUVs: surface charge density and the nature of counterions (buffer).

Our first finding is that a critical density of surface charge groups is necessary for the rupture of adsorbed SUVs and the formation of stable SPBs from charge neutral (zwitterionic) lipids. This is found for either positively or negatively charged surfaces. Fig. 2 shows a set of FRAP experiments for positively charged surfaces. In this experiment, mixed SAMs on gold are formed from mixed NH_2-$(CH_2)_{11}$-SH and HO-$(CH_2)_{11}$-SH (see Fig. 1) solutions. Each SAM-covered surface is incubated for ≥1 h with an SUV solution of 1 mM eggPC lipid (doped with 5% TR-DHPE) in Tris buffer, which contains the positively charged molecular ion $C_3H_5(OH)_3$ $-NH_3^+$. After thorough rinsing with the buffer solution, each surface is imaged under a fluorescence microscope. On surfaces with ≥80% $-NH_3^+$ groups, the lipid molecules are clearly in a fluidic state, indicating the formation of supported lipid

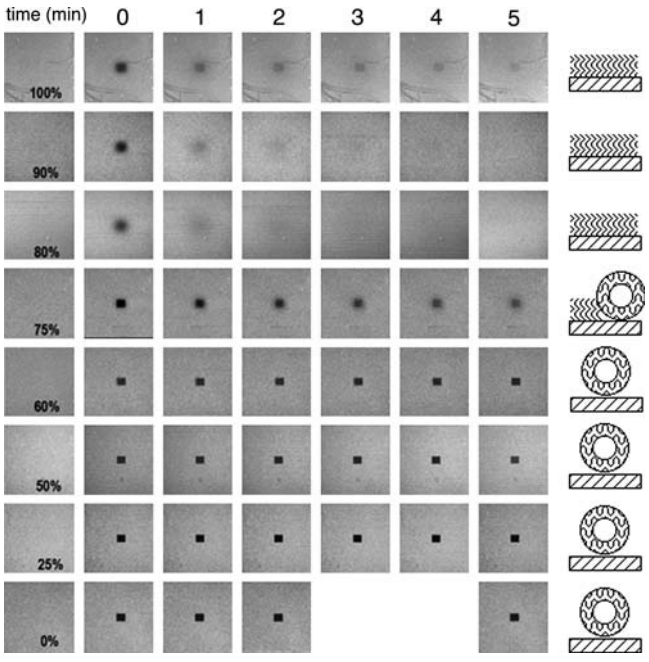

FIGURE 2 FRAP images obtained after the adsorption of eggPC SUVs on surfaces of mixed thiol SAMs ($-NH_3^+$ and $-OH$ terminated). The images before photobleaching (*left*) are labeled with mole percentages of $-NH_3^+$ in the SAMs. After photobleaching at time zero, images are taken at 1-min time intervals. The size of each photobleached area is 16 × 16 μm, and the size of each image is 160 × 160 μm. Cartoons on the right-hand side illustrate supported phospholipids bilayers for 100–80% $-NH_3^+$ and adsorbed vesicles for 60–0% $-NH_3^+$. Tris buffer was used in these experiments.

bilayers on the surface. The majority of fluorescence intensity is recovered in 5 min. In contrast, surfaces with <70% NH_3^+ show no fluorescence recovery. Thus, the adsorbed vesicles do not rupture on these surfaces to form continuous SPBs. The surface with 75% NH_3^+ is an intermediate case and partial fluorescence recovery is observed. Fig. 3 shows quantitative fluorescence recovery 5 min after bleaching for the surface $-NH_3^+$ concentrations investigated. There is clearly a phase transition at a critical surface coverage of $\theta_C = 75\%$.

Similar results are obtained for negatively charged surfaces from mixed SAMs of HOOC-$(CH_2)_{11}$-SH and HO-$(CH_2)_{11}$-SH on Au. In this experiment, the SUV solution contains PBS buffer with negatively charged molecular ions (HPO_4^{2-} and $H_2PO_4^-$). We again see a phase transition at a surface $-COO^-$ coverage of ~70%, above which adsorbed SUVs rupture and form a stable SPB on the surface. For $-COO^-$ coverage below ~70%, adsorbed SUVs do not rupture to form SPB. Two representative sets of FRAP images below and above the critical surface $-COO^-$ coverage are shown in Fig. 4.

We quantitatively analyze the FRAP data using the reported method (21) to extract lateral diffusion coefficients for the lipids. The resulting diffusion coefficients on different SAM surfaces are summarized in Table 1. As a reference, we find that SPBs readily form on a clean glass surface from SUVs, in agreement with previous findings (22); the lateral diffusion coefficient extracted from FRAP images (not shown) is 2.8 $\pm$ 0.5 $\times$ 10^{-8} cm^2/s, which is close to the literature value (21). We make two observations from the data in Table 1: i), On either positively ($-NH_3^+$) or negatively ($-COO^-$) charged surfaces, lipid molecules are completely immobile when the mole fraction (χ) of surface charge groups is <70%; and ii), on surfaces where SPBs are formed ($\chi = 1 - 0.8$), the lipid mobility increases slightly as χ decreases but all are slightly lower than the diffusion coefficient for the SPB on clean glass. We believe there is attractive interaction between surface charge groups and zwitterions lipids, and a critical

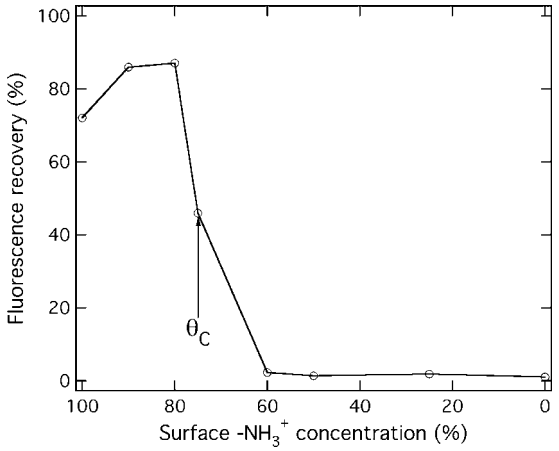

FIGURE 3 Recovery of fluorescence intensity in the photobleached area after 5 min for the surfaces shown in Fig. 2.

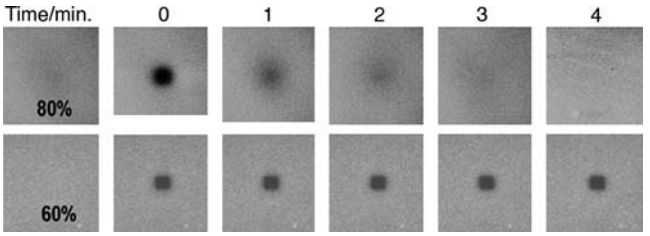

FIGURE 4 FRAP images obtained after the adsorption of eggPC SUVs on surfaces of mixed SAMs ($-COO^-$ and $-OH$ terminated thiols) with mole percentages of $-COO^-$ shown on the left. Images are taken at 0–4 min after photobleaching. PBS buffer was used. The size of each photobleached area is ~16 $\times$ 16 μm.

concentration of surface charge groups (for $\chi > 70\%$) is necessary to induce a phase transition from adsorbed vesicles to SPBs. The attractive interaction between surface charge groups and zwitterions lipids also decreases lipid mobility.

The second important finding from this study is the critical role of buffer type on SUV adsorption and SPB formation on the positively or negatively charged SAM surfaces. As shown above, SUVs of zwitterionic eggPC lipids readily rupture and form SPBs on either 100% $-NH_3^+$ or 100% $-COO^-$ functionalized SAMs. In the case of $-NH_3^+$ terminated surface, the buffer solution used is Tris ($C_3H_5(OH)_3 - NH_3^+ + Cl^-$), whereas for $-COO^-$ terminated surface, the buffer is PBS ($HPO_4^{2-} + Na^+$ and $H_2PO_4^- + Na^+$). When we switch these two types of buffers, PBS for the $-NH_3^+$ terminated surface and Tris for the $-COO^-$ surface, we find that the adsorbed vesicles do not rupture, as shown by FRAP results in Fig. 5.

We arrive at two conclusions: 1), Electrostatic interaction is critical in the rupturing of adsorbed zwitterionic vesicles and the formation of a stable supported phospholipid bilayer. On the surface of functional thiol SAM, a critical surface coverage of ~75% (or ~3 $\times$ $10^{14}/cm^2$) of surface charged groups ($-NH_3^+$ or $-COO^-$) is needed; and 2), the nature (size) of counterions near the charged surface affects the interaction between vesicles and the surface. For positive charged $-NH_3^+$ surface in Tris buffer, the counterions near the surface are small atomic ions, Cl^-. There is sufficient interaction between the zwitterionic lipids and the surface $-NH_3^+$ groups to result in vesicle rupture and SPB formation. When the buffer is PBS, the counterions near the

TABLE 1 Summary of lateral diffusion coefficients on lipids on SAM-covered surfaces

Mol fraction of AUT in the mixed SAM	Diffusion coefficient (10^{-8} cm^2/s)	Mol fraction of CDT in the mixed SAM	Diffusion coefficient (10^{-8} cm^2/s)
1	1.7 ± 0.3	1	1.8 ± 0.2
0.9	2.3 ± 0.8	0.9	2.0 ± 0.4
0.8	2.3 ± 0.9	0.8	2.1 ± 0.3
0.75	0.27 ± 0.04	0.7	0.25 ± 0.08
≤0.6	<0.001	≤0.6	<0.001

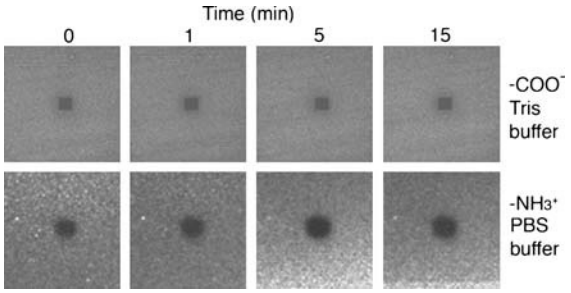

FIGURE 5 FRAP images obtained after the adsorption of eggPC SUVs on 100% $-COO^-$ or 100% $-NH_3^+$ terminated thiol SAMs. Images are taken at 0–15 min after photobleaching. PBS buffer was used for $-NH_3^+$ and Tris buffer for $-COO^-$ terminated surfaces.

surface are larger molecular ions, HPO_4^{2-} and $H_2PO_4^-$. The presence of these molecular ions seems to reduce the interaction between vesicles and the $-NH_3^+$ surface, and vesicle rupture does not occur.

The critical role of counterion type in the buffer solution was only seen in the interaction of zwitterionic lipid vesicles with charged molecular surfaces. When DOTAP, positively charged lipid vesicles, adsorb onto the negatively charged molecular surface (CDT SAM), SPBs with lateral lipid mobility are observed in either Tris (Fig. 6) or PBS buffer (data not shown) for $\chi \geq 60\%$. On the other hand, no adsorption of DOTAP occurred on the positively charged molecular surfaces. It indicates that, in the case of charged lipid vesicles, the electrostatic interaction between the positively charged vesicles and negatively charged surface is strong enough and counterions have little effect.

A mechanistic picture emerging from the above results is illustrated in Fig. 7. A charge neutral lipid vesicle with zwitterionic headgroups can interact with a charged surface

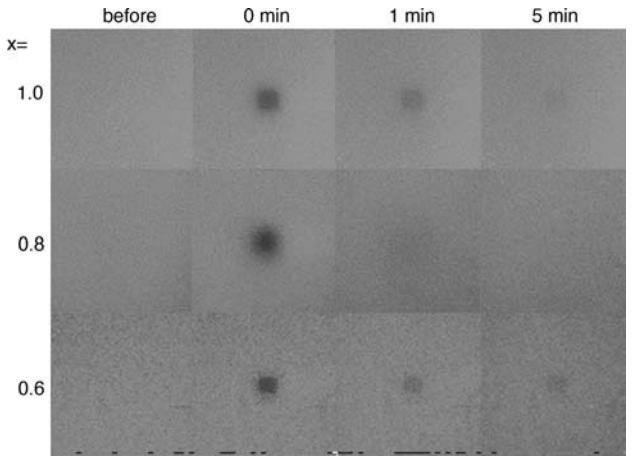

FIGURE 6 FRAP images taken after the adsorption of SUVs of DOTAP (positively charged lipid) in Tris buffer on the surface of mixed SAMs ($-COO^-$ and $-OH$ terminated thiols) with mole percentages of $-COO^-$ shown on the left. Images are taken at 0–5 min after photobleaching. Similar results are obtained for PBS buffer.

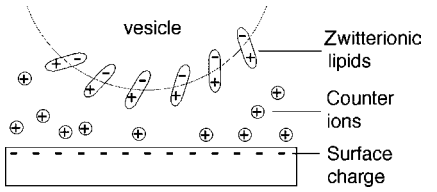

FIGURE 7 Schematic illustration of a vesicle with zwitterionic headgroups interacting with a charged surface in the presence of counterions in the solution phase.

through charge-dipole interaction. In addition to the universally present van der Waals forces, the attractive interaction can come from the positively charged choline group in a lipid molecule with negative surface charge groups on the substrate surface (or from the negatively charged phosphate group with positively charged surface). This attractive interaction induces surface tension on the vesicle and, when sufficiently high, leads to the rupture of adsorbed vesicles and the formation of supported phospholipid bilayer. However, charge neutrality of the whole interface region must be maintained during such interaction. This means that counterions must be present. These counterions must be squeezed into a smaller and smaller space as the two interfaces approach. This is the well-known entropic or osmotic repulsion, the origin of electric double layer interaction (23). Such an entropic repulsion should depend strongly on the size of the counterion based on simple phase space argument: the larger the size of the counterion, the higher the entropic repulsion. When the entropic repulsion is sufficiently high, lipid-surface interaction is weakened and vesicle rupture does not occur. This is exactly what we observe for effects of molecular counterions.

CONCLUSIONS

We report the critical role of surface charge density in controlling the rupture of adsorbed zwitterionic lipid vesicles to form supported phospholipid bilayers. On either positively ($-NH_3^+$) or negatively ($-COO^-$) charged SAM surfaces, there is a critical surface charge density of 75% (or $\sim 3 \times 10^{14}/cm^2$) above which charge neutral eggPC vesicles readily fuse into SPBs. Unlike that between charged lipids and oppositely charged surfaces, the interaction between zwitterionic lipids and charged surfaces depends critically on the type of counterions in the solution (buffer). Rupturing of adsorbed vesicles to form SPBs on each charged molecular surface occurs only when the molecular ion of the buffer used is of the same charge type. We attribute this to the increased entropic repulsion due to the large size of molecular counterions. These results establish the critical role of electrostatic interaction between zwitterionic charge neutral lipids and surface charge groups in inducing the transition from adsorbed vesicles to supported phospholipid bilayers. Controlling surface charge density may be a general strategy

to form stable supported phospholipid bilayers as models in biological studies or in applications such as biosensors or membrane protein microarrays.

We thank Prof. Tim Lodge and Dr. Joona Bang for help with light scattering measurements.

This work was supported in part by a National Science Foundation SBIR-I grant (0318938) to MicroSurfaces.

REFERENCES

1. Elender, G., M. Kuhner, and E. Sackmann. 1996. Functionalization of Si/SiO2 and glass surfaces with ultrathin dextran films and deposition of lipid bilayers. *Biosens. Bioelectron.* 11:565–577.
2. Groves, J. T., and S. G. Boxer. 2002. Micropattern formation in supported lipid membranes. *Acc. Chem. Res.* 35:149–157.
3. Sackmann, E. 1996. Supported membranes: scientific and practical applications. *Science.* 271:43–48.
4. Stora, T., Z. Dienes, H. Vogel, and C. Duschl. 2000. Histidine-tagged amphiphiles for the reversible formation of lipid bilayer aggregates on chelator-functionalized gold surfaces. *Langmuir.* 16:5471–5478.
5. Reimhult, E., F. Hook, and B. Kasemo. 2003. Intact vesicle adsorption and supported biomembrane formation from vesicles in solution: influence of surface chemistry, vesicle size, temperature, and osmotic pressure. *Langmuir.* 19:1681–1691.
6. Richter, R., A. Mukhopadhyay, and A. Brisson. 2003. Pathways of lipid vesicle deposition on solid surfaces: a combined QCM-D and AFM study. *Biophys. J.* 85:3035–3047.
7. Wagner, M. L., and L. K. Tamm. 2000. Tethered polymer-supported planar lipid bilayers for reconstitution of integral membrane proteins: silane-polyethyleneglycol-lipid as a cushion and covalent linker. *Biophys. J.* 79:1400–1414.
8. Reimhult, E., F. Hook, and B. Kasemo. 2002. Vesicle adsorption on SiO2 and TiO2: dependence on vesicle size. *J. Chem. Phys.* 117:7401–7404.
9. Baumgart, T., and A. Offenhausser. 2003. Polysaccharide-supported planar bilayer lipid model membranes. *Langmuir.* 19:1730–1737.
10. McArthur, S. L., M. W. Halter, V. Vogel, and D. G. Castner. 2003. Covalent coupling and characterization of supported lipid layers. *Langmuir.* 19:8316–8324.
11. Holden, M. A., S.-Y. Jung, T. Yang, E. T. Castellana, and P. S. Cremer. 2004. Creating fluid and air-stable solid supported lipid bilayers. *J. Am. Chem. Soc.* 126:6512–6513.
12. Cremer, P. S., and S. G. Boxer. 1999. Formation and spreading of lipid bilayers on planar glass supports. *J. Phys. Chem. B.* 103:2554–2559.
13. Ekeroth, J., P. Konradsson, and F. Hook. 2002. Bivalent-ion-mediated vesicle adsorption and controlled supported phospholipid bilayer formation on molecular phosphate and sulfate layers on gold. *Langmuir.* 18:7923–7929.
14. Reviakine, I., A. Simon, and A. Brisson. 2000. Effect of Ca2+ on the morphology of mixed DPPC-DOPS supported phospholipid bilayers. *Langmuir.* 16:1473–1477.
15. Langner, M., and K. Kubica. 1999. The electrostatics of lipid surfaces. *Chem. Phys. Lipids.* 101:3–35.
16. Nuzzo, R. G., and D. L. Allara. 1983. Adsorption of bifunctional organic disulfides on gold surfaces. *J. Am. Chem. Soc.* 105:4481–4483.
17. Ulman, A. 1991. An Introduction to Ultrathin Organic Films. Academic Press, Boston.
18. Steinem, C., A. Janshoff, W.-P. Ulrich, M. Sieber, and H.-J. Galla. 1996. Impedance analysis of supported lipid bilayer membranes: a scrutiny of different preparation techniques. *Biochim. Biophys. Acta.* 1279:169–180.
19. Plant, A. L. 1999. Supported hybrid bilayer membranes as rugged cell membrane mimics. *Langmuir.* 15:5128–5135.
20 Arnold, R., W. Azzam, A. Terfort, and C. Wöll. 2002. Preparation, modification, and crystallinity of aliphatic and aromatic carboxylic acid terminated self-assembled monolayers. *Langmuir.* 18:3980–3992.
21. Berquand, A., P.-E. Mazeran, J. Pantingny, V. Proux-Delrouyre, J.-M. Laval, and C. Bourdillon. 2003. Two-step formation of streptavidin-supported lipid bilayers by PEG-triggered vesicle fusion. Fluorescence and atomic force microscopy characterization. *Langmuir.* 19:1700–1707.
22. Brian, A. A., and H. M. McConnell. 1984. Allogeneic stimulation of cytotoxic T cells by supported planar membranes. *Proc. Natl. Acad. Sci. USA.* 81:6159–6163.
23. Israelachvili, J., and H. Wennerstrom. 1996. Role of hydration and water structure in biological and colloidal interactions. *Nature.* 379: 219–225.